0013-7227/82/1103-0773$02.00/0
Endocrinology

Vol. 110, No. 3
Printed in U.S.A.

Properties of the Hormonally Responsive Rabbit Luteal Adenylyl Cyclase: Effects of Guanine Nucleotides and Magnesium Ion on Stimulation by Gonadotropin and Catecholamines*

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ABSTRACT. The effects of guanine nucleotides and magnesium on rabbit luteal adenylyl cyclase were studied in the absence and presence of LH and isoproterenol. Adenylyl cyclase was activated by guanyl-5′-yl imidodiphosphate [GMP-P(NH)P], guanyl-5′-yl diphosphonate [GMP-P(CH_2)P], and guanosine 5′-(3-*O*-thio) triphosphate (GTPγS) to differing degrees and with time courses of cAMP accumulation that exhibited distinct and differing lags, and by GTP without a lag. Steady state activities obtained in the presence of GMP-P(NH)P and GTPγS were about 6–7 times that obtained with GTP. The rate of activation by GMP-P(NH)P could be accelerated in two ways: 1) increasing the concentration of Mg ion in the medium; and 2) the addition of either LH or isoproterenol. Stimulation of rabbit luteal enzymatic activity by LH and isoproterenol showed a stringent requirement for both guanine nucleotides and Mg ion. In the presence of GTP, hormonal stimulations by either LH or isoproterenol were optimal (up to 12-fold) only over a very narrow range of Mg ion concentration. In the presence of 0.1 mM ATP and 1.0 mM EDTA, maximal hormonal stimulation was obtained at 0.6 mM total added $MgCl_2$, and changes in the concentration of total added $MgCl_2$ by as little as 0.1 mM resulted in drastic decreases in the degree of stimulation by either hormone. The addition of substances which reduce the concentration of free Mg ions and act as Mg buffers in the 10- to 100-μM range, such as ATP and inorganic phosphate, resulted in an increase in the range of added $MgCl_2$ that promoted optimal hormonal stimulation. The concentrations of LH and isoproterenol that stimulate rabbit luteal adenylyl cyclase half-maximally (K_{act} values) were dependent on the type of guanine nucleotide present at the time of assay. K_{act} values for both LH and isoproterenol were higher in the presence of 10 μM GTP than in the presence of 10 μM GMP-P(NH)P. Adenylyl cyclase activities elicited by LH and isoproterenol were not additive in the presence of GMP-P(NH)P, indicating that these two hormones activate the same adenylyl cyclase system. It is concluded that both LH and isoproterenol stimulate the rabbit luteal adenylyl cyclase through a similar if not identical coupling process, that this process resembles in its stringent dependencies those described for other mammalian adenylyl cyclases, and that previously observed effects of 1.5–3.0 mM ATP, leading to higher relative stimulations of LH, are due to the Mg-buffering capacity of ATP, as opposed to being due to unique nucleotide-specific requirements in the gonadotropin effect on corpus luteum adenylyl cyclase. (*Endocrinology* **110:** 773, 1982)

MUCH new information has been obtained recently on the hormonal regulation of adenylyl cyclases. It is now clear that the signal transduction process intervening between receptor occupancy by hormone and stimulation of ATP to cAMP cyclizing activity is critically controlled by guanine nucleotides. Hence, in well defined systems, development of hormonal stimulation exhibits an absolute requirement for either GTP or one of its analogs (1, 2). Corpora lutea of rabbits and rats (3–5) as well as other species (6–8) respond to the gonadotropin LH, to prostaglandins (PGs), and to catecholamines through stimulation of adenylyl cyclase activity. In rabbit corpus luteum membranes, we have recently observed that the guanine nucleotide requirements for PG and prostacyclin stimulation of adenylyl cyclase do not differ substantially from those seen in other nonovarian adenylyl cyclase systems (9). However, studies on the requirements of this luteal adenylyl cyclase for optimal responsiveness to LH showed that relative stimulation by the gonadotropin was consistently higher when assay medium contained high (millimolar) levels of ATP than when ATP was in the so-called low concentration range of 0.1 mM (3, 4). The difference in stimulation of activity was notable: 5- to 10-fold when determined using 3.0 mM ATP *vs.* 1.5- to at most 2.5-fold when assayed at 0.1 mM ATP. This difference was not due to the lack of GTP in the incubation medium (3). On the basis of the enhancing effect of ATP, which appeared to be selective for LH, we concluded in previous studies that the LH-mediated stimulation of adenylyl cyclase might proceed

Received July 2, 1981.
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* This work was supported in part by Grants AM-19318, HD-09581, and HD-07945 from the NIH.
† Recipient of NIH Research Service Award HD-05757.

through a rather unique transduction process different *in toto* or in part from that used by prostaglandins (3, 4). This conclusion is in sharp contrast to what one would expect on the basis of the well known two-component arrangement of adenylyl cyclases (1), where the only known variables affecting stimulation of cyclizing activity are guanine nucleotides and Mg ion, and the nucleotide requirement for receptor-cyclase coupling cannot be satisfied by ATP. In recent studies, we noted that the stimulation of rabbit luteal adenylyl cyclase by PGs and LH are partially additive (Abramowitz, J., and L. Birnbaumer, unpublished). This suggested the existence of more than one adenylyl cyclase system: one apparently responsive to both LH and PGs, and the other possibly responding only to PGs. On the other hand, preliminary experiments on catecholamine responsiveness of the rabbit luteal adenylyl cyclase showed that LH and isoproterenol stimulations are not additive. Yet, the characteristics of catecholamine responsiveness in luteal membranes have not been studied.

In early studies with fat cell ghosts (10), as well as in recent experiments from this laboratory (Iyengar, R., and L. Birnbaumer, submitted) with other adenylyl cyclase systems, such as those of liver membranes and S49 cells, it was shown that not only guanine nucleotides but also Mg ion play a critical role in determining the relative stimulation that can be elicited at any given guanine nucleotide concentration. This prompted us to reexamine the apparent ATP requirement for optimal LH stimulation in the luteal adenylyl cyclase system in terms of what changing ATP concentrations would do to the levels of free Mg ion in the incubation mixture and to explore whether the changes in Mg ion levels caused by the Mg-binding capacity of ATP could account for the observed changes in LH responsiveness. To determine if the effects of LH on luteal adenylyl cyclase activity were specific, we conducted parallel studies using isoproterenol and compared the effects of the two hormones. In carrying out this study, we used well washed membrane particles and special reagents treated to remove contaminating guanine nucleotides and guanine nucleotide-like materials. Preliminary accounts of some of these findings have been presented (11, 12).

Materials and Methods

Materials

Inorganic ^{32}P was purchased from Union Carbide (Tuxedo, NY). [^{3}H]cAMP (10–20 Ci/mmol) was obtained from Schwarz/Mann (Orangeburg, NY). Guanyl-5′-yl imidodiphosphate [GMP-P(NH)P] and guanosine 5′-(3-*O*-thio)triphosphate (GTPγS) were purchased from Boehringer-Mannheim (Mannheim, West Germany), and guanyl-5′-yl diphosphonate [GMP-P(CH$_2$)P] was obtained from International Chemical and Nuclear Corp. (Irvine, CA). ATP (Na-salt; catalogue no. A-2383), GTP, cAMP, EDTA, Tris, creatine phosphate, and myokinase (2000 U/mg) were purchased from Sigma Chemical Co. (St Louis, MO). Creatine phosphokinase (200 U/mg) was obtained from Calbiochem (LaJolla. CA). The LH (NIH-LH-S19) and highly purified hCG (hCG-CR119) were obtained from the NIH. (−)Isoproterenol was a gift from Dr. Nachod of Sterling-Wintrop Laboratories (Rennselear, NY). PGE$_1$ was a gift from Dr. John E. Pike of The Upjohn Co. (Kalamazoo, MI). The hCG used to induce pseudopregnancy was a gift from Dr. John B. Jewell of Ayerst Laboratories (New York, NY). When required, the components of the nucleoside triphosphate-regenerating system used in the adenylyl cyclase assay were subjected to purification steps to decrease contamination with guanine nucleotide-like compounds, as recently described (13). All other chemicals and reagents were of the highest commercially available purity and were used without further purification.

[α-^{32}P]ATP (SA, >50 Ci/mmol) was synthesized according to the method of Walseth and Johnson (14) and was purified by DEAE-Sephadex A-25 chromatography, as described previously (15). [α-^{32}P]ATP prepared by this method was supplied by the Core Laboratory on Cyclic Nucleotide Research, Center for Population Research and Studies on Reproductive Biology, Baylor College of Medicine (Houston, TX).

Animals

New Zealand White rabbits (3.0–4.5 kg) were used throughout. Pseudopregnancy was induced by injection of 100 IU hCG (Ayerst) in saline, iv. The rabbits were killed by cervical dislocation on day 7 of pseudopregnancy (the day of hCG injection was day 0). The ovaries were removed and placed in ice-cold Krebs-Ringer bicarbonate, pH 7.4, until dissection of the corpora lutea. The dissected corpora lutea were homogenized, and membrane particles were prepared as previously described (4).

Adenylyl cyclase assays

Adenylyl cyclase activity was determined at 32.5 C in medium containing the indicated concentration of ATP (with 1–5 × 10^7 cpm [α-^{32}P]ATP), the indicated concentration of $MgCl_2$, 1.0 mM EDTA, 1.0 mM cAMP (with ~10,000 cpm [^{3}H]cAMP), 20 mM creatine phosphate, 0.2 mg/ml creatine kinase, 0.02 mg/ml myokinase, and 25 mM Tris-HCl, pH 7.5. When present and unless indicated otherwise, the following concentrations were used: LH, 10 μg/ml; hCG, 10 μg/ml; (−)isoproterenol, 100 μM; PGE$_1$, 10 μg/ml; GTP, 10 or 100 μM; and GMP-P(NH)P, 10 or 100 μM. Fixed time assays were conducted in a final volume of 50 μl and were carried out for 10 min. These assays were stopped by the addition of 100 μl stopping solution consisting of 10 mM cAMP, 40 mM ATP, and 1% sodium dodecyl sulfate. Time courses of cAMP accumulation were carried out by incubating larger volumes, removing 50-μl aliquots at the indicated times, and stopping the reaction by the addition to 100 μl of the above-described stopping solution. The [^{32}P]cAMP formed and the [^{3}H]cAMP added to monitor recovery were isolated according to the method of Salomon *et al.* (16) using Dowex and alumina chromatography, as modified by Bockaert *et al.* (17). All experiments were repeated at least three times.

and representative results are presented.

Protein was determined by the method of Lowry *et al.* (18) using crystalline bovine serum albumin (fraction V) as standard.

Results

Guanine nucleotide activation of luteal adenylyl cyclase

The characteristics of the activation of luteal adenylyl cyclase by guanine nucleotides have not been studied. We examined the effects of various GTP analogs as well as of GTP itself on the time courses of cAMP accumulation. Each guanine nucleotide tested stimulated enzymatic activity with distinct characteristics (Fig. 1). Stimulation by GTP was rapid and demonstrated no detectable lag in the progress curve. In fact, GTP stimulated adenylyl cyclase activity with a slight burst, a phenomenon that has been observed in other adenylyl cyclases as well (19). In contrast, all three hydrolysis-resistant GTP analogs stimulated enzymatic activity slowly, with distinct lags in their course of stimulation. Under the assay conditions used (0.11 mM ATP and 5.0 mM $MgCl_2$), the stimulation by the analogs required between 6 and 10 min in order to reach steady state. Steady state activities obtained in the presence of GMP-P(NH)P and GTPγS were about 6–7 times those obtained with GTP, while steady state activities obtained with GMP-P(CH_2)P were about 3 times greater than those obtained with GTP. GTPγS elicited the greatest degree of stimulation and was the fastest stimulating guanine nucleotide analog tested.

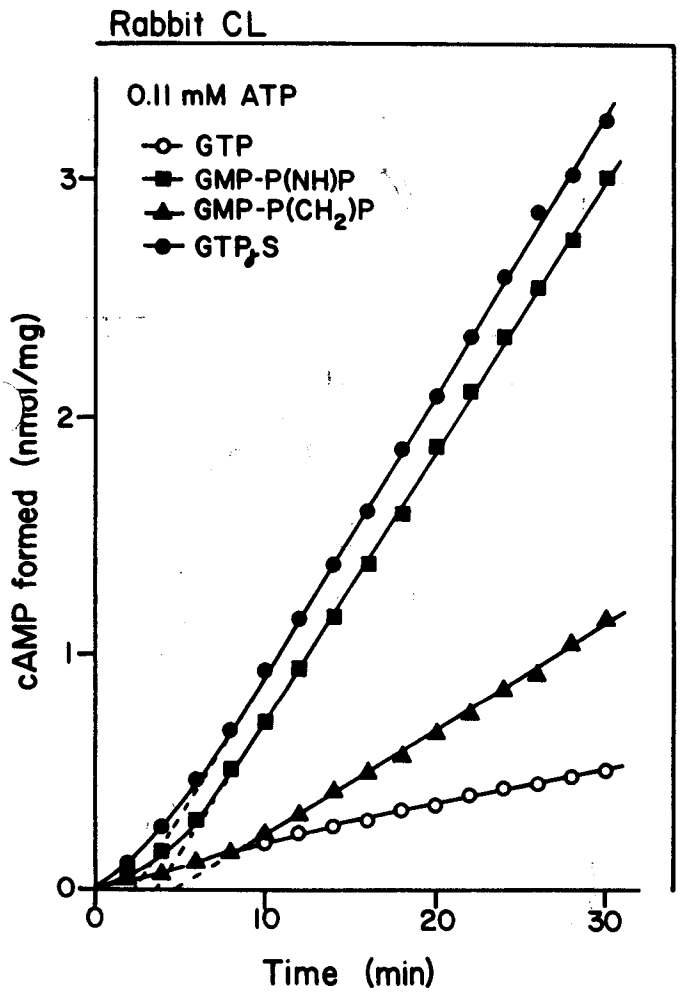

FIG. 1. Time courses of activation of rabbit luteal adenylyl cyclase by GTP and GTP analogs. Rabbit corpus luteum membranes (130 μg protein/ml) were incubated in a final volume of 1.25 ml, as described in *Materials and Methods*, using 0.11 mM ATP, 5.0 mM $MgCl_2$, and 1 mM EDTA. At the indicated time intervals, 50-μl aliquots were withdrawn, and [^{32}P]cAMP accumulated was determined. All four guanine nucleotides were present at a concentration of 10 μM each. For the rest of the conditions, see *Materials and Methods*. In this and subsequent figures, single representative experiments are shown; each experiment was repeated three times.

Effect of Mg on the time-dependent activation of luteal adenylyl cyclase by GMP-P(NH)P

In view of the reports of Alvarez and Bruno (20) indicating that activation of cardiac adenylyl cyclase by GMP-P(NH)P is dependent on Mg ion, we explored whether this is also true for the luteal adenylyl cyclase. In initial experiments not reported here, we indeed found that prior incubation of luteal membranes with 100 μM GMP-P(NH)P, followed by assay for adenylyl cyclase activity at 5 mM $MgCl_2$, had no effect on the time course of cAMP accumulation, *i.e.* a lag was present as if no prior incubation had been carried out. However, if $MgCl_2$ was included during the preliminary incubation (15 min at 32.5 C), the lag observed in the subsequent adenylyl cyclase assay was abolished. Therefore, we explored in direct incubations the effects of varying concentrations of $MgCl_2$ on the activation of the luteal adenylyl cyclase as seen in time-course studies. Figure 2 illustrates the results of such an experiment. It may be seen that as the Mg ion concentration in excess of added ATP and EDTA was increased from 0.1 to 10.0 mM, the lags observed in the progress curves were progressively reduced and reached a limiting value. Thus, the time required to reach steady state velocity was reduced from 15 to 6 min in the presence of 0.1 and 5.0–10 mM Mg ion in excess of ATP and EDTA, respectively. In addition to reducing the time required to reach steady state activity, increasing the Mg ion concentration resulted in a concentration-dependent increase in the steady state activity observed in the presence of 10 μM GMP-P(NH)P (Fig. 2, *inset*). The concentration of Mg ion in excess of added ATP and EDTA required to half-maximally stimulate adenylyl cyclase activity in the presence of GMP-P(NH)P at steady state was 0.9 mM. This value is similar to the Mg ion concentration required to half-maximally activate rabbit luteal adenylyl cyclase activity in the presence of

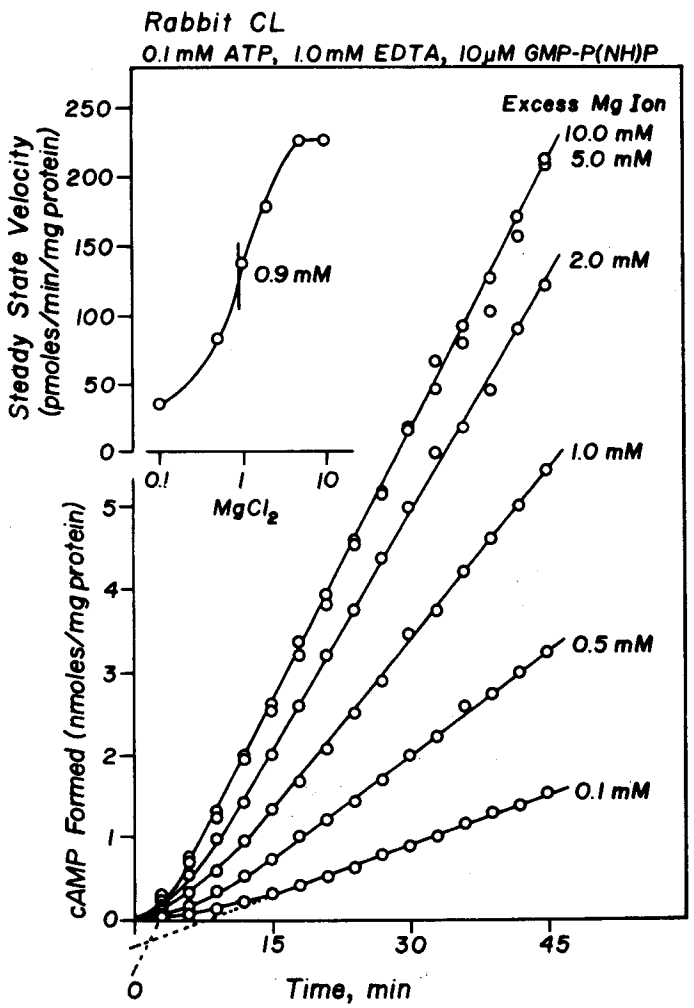

FIG. 2. Effects of varying concentrations of Mg ion on the rate of activation of the rabbit luteal adenylyl cyclase by GMP-P(NH)P. Rabbit corpus luteum membranes (32 μg/ml) were incubated in a final volume of 2.0 ml, as described in *Materials and Methods*, in the presence of 10 μM GMP-P(NH)P. Mg ion concentrations are shown in excess of added ATP (0.1 mM) and EDTA (1.0 mM). At 3-min intervals, 50-μl aliquots were withdrawn, and [^{32}P]cAMP accumulated was determined. For the rest of conditions, see *Materials and Methods*.

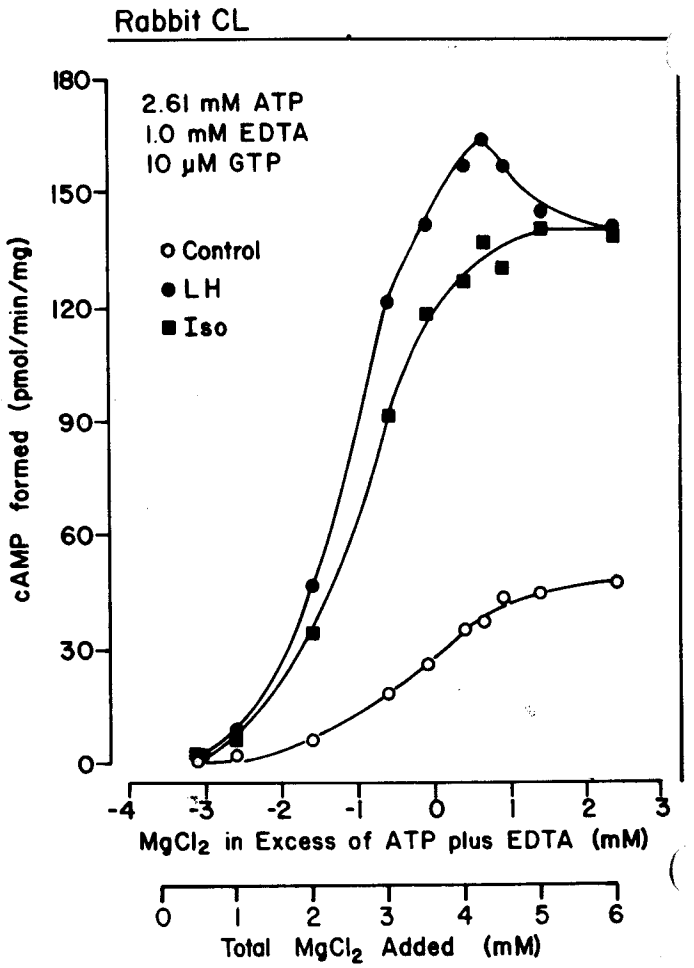

FIG. 3. Effects of varying concentrations of Mg ion on rabbit luteal adenylyl cyclase activity. Activities were determined at 32.5 C for 10 min in the presence of 2.61 mM ATP and 10 μM GTP without (○) and with 10 μg/ml LH (●) or 100 μM isoproterenol (■), as described in *Materials and Methods*. The membrane protein concentration was 29.3 μg/50-μl assay. Mg concentrations are presented in two ways: total $MgCl_2$ added, and Mg in excess of added ATP plus EDTA. For the rest of the conditions, see *Materials and Methods*.

10 μM GMP-P(NH)P when enzyme activities were obtained in a 0- to 10-min assay, *i.e.* under presteady state conditions (9).

Effects of Mg on the responsiveness of luteal adenylyl cyclase to LH and isoproterenol

Mg ion is not only important in the regulation of the guanine nucleotide sensitivity of adenylyl cyclase, but also in the regulation of hormonal stimulation of the enzyme system. Figure 3 depicts the effects of varying concentrations of $MgCl_2$ on basal, LH-stimulated, and isoproterenol-stimulated adenylyl cyclase activity assayed in the presence of 2.61 mM ATP and 10 μM GTP. Increasing the total $MgCl_2$ concentration from 0.5 to 6.0 mM resulted in a concentration-dependent increase in basal activity. Absolute enzymatic activity in the presence of hormone also increased with increasing $MgCl_2$ concentration. Maximal adenylyl cyclase activity in the presence of LH was obtained at 4.25 mM total $MgCl_2$. Further increases in the $MgCl_2$ concentration resulted in a decrease in enzymatic activity obtained in the presence of LH. Maximal enzymatic activity in the presence of isoproterenol was obtained at a similar $MgCl_2$ concentration; however, further increases in the $MgCl_2$ concentration did not result in decreased enzymatic activity.

The degree of hormonal stimulation elicited by LH and isoproterenol in the presence of GTP was optimal over an extremely narrow range of Mg ion concentrations. This is illustrated in Fig. 4 under three different assay conditions. In the presence of 0.1 mM ATP and 1

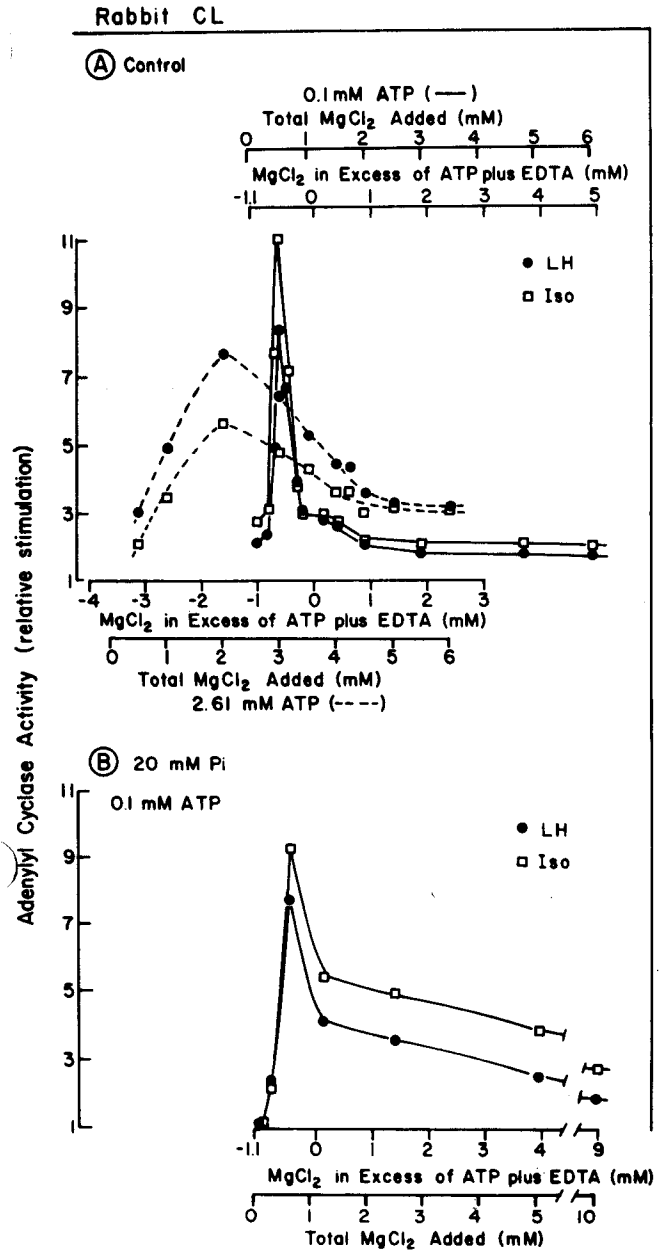

FIG. 4. Stringency in the Mg ion concentration required for optimal hormonal stimulation of rabbit luteal adenylyl cyclase. Assays were determined at 32.5 C for 10 min in the presence of 1.0 mM EDTA, 10 μM GTP, and the indicated concentrations of ATP, inorganic phosphate (Pi), and $MgCl_2$. When present, the LH level was 10 μg/ml, and the isoproterenol concentration was 100 μM. The membrane protein contents were 15.5 μg/50-μl assay (A; ——), 29.3 μg/50-μl assay (A; - - -), and 11.8 μg/50-μl assay (B). Mg concentrations are presented in two ways: total $MgCl_2$ added, and Mg in excess of added ATP plus EDTA. Relative stimulations due to hormone addition were calculated by dividing each experimental value by its appropriate control value. For the rest of the assay conditions, see *Materials and Methods*.

μM GTP, hormonal stimulation due to LH and isoproterenol was maximal at a total $MgCl_2$ concentration of 0.6 mM. Changes in the $MgCl_2$ concentration as small as 0.1 mM resulted in a drastic decrease in the degree of hormonal stimulation in response to both LH and isoproterenol (Fig. 4A, ——). In fact, this absolute value of the added $MgCl_2$ concentration that would yield the maximum response to LH and isoproterenol was very difficult to reproduce. It varied up to 0.2 mM units from membrane to membrane batch and up to 0.1 mM units from experiment to experiment. The same experiment was repeated, except that the ATP concentration was increased to 2.61 mM (Fig. 4A, - - -). Under these assay conditions, hormonal stimulation due to LH and isoproterenol was obtained over a wider range of $MgCl_2$ concentrations. It was reasoned that this effect of ATP might be due to its Mg-binding ability (21), which would lead to a decrease in the concentration of free Mg ions in the assay medium. In agreement with this assumption, the addition of 20 mM inorganic phosphate, which also binds Mg, resulted in an increase in the range of $MgCl_2$ concentrations that would support hormonal stimulation by both LH and isoproterenol at 0.1 mM ATP (Fig. 4B). Thus, it appears that the addition of substances to the assay that reduce the concentration of free Mg ions and act as Mg buffers will result in an increase in the range of $MgCl_2$ concentrations which will promote hormonal stimulation of rabbit luteal adenylyl cyclase activity by both LH and isoproterenol. However, the reduction in the Mg ion concentration will also be accompanied by a decrease in catalytic activity (Fig. 3). It should be noted that although the concentration ranges of Mg ion at which stimulation by LH and isoproterenol relative to basal activity was maximal were the same for both stimulating hormones, the stimulatory effect of the catecholamine was less inhibited by Mg ion in excess of the optimal range than was the effect of LH. As a consequence, at high $MgCl_2$ concentrations, LH responsiveness is almost absent, while catecholamine responsiveness, although largely reduced with respect to its potential maximum, is still readily demonstrable.

Effects of guanine nucleotides on LH and isoproterenol stimulation of luteal adenylyl cyclase

One property that appears to be common to all adenylyl cyclase systems is the requirement for guanine nucleotides in order to obtain hormonal stimulation (1, 2). Thus, assay conditions employed previously using high levels of ATP and nucleoside triphosphate-regenerating systems that are now known to have been contaminated with significant concentrations of guanine nucleotides had not permitted the detection of such guanine nucleotide requirements for LH stimulation, even though the system had not been saturated with guanine nucleotide, as seen by an up to 2-fold stimulation of basal activity (3, 4). Using reagents purified by ion exchange chroma-

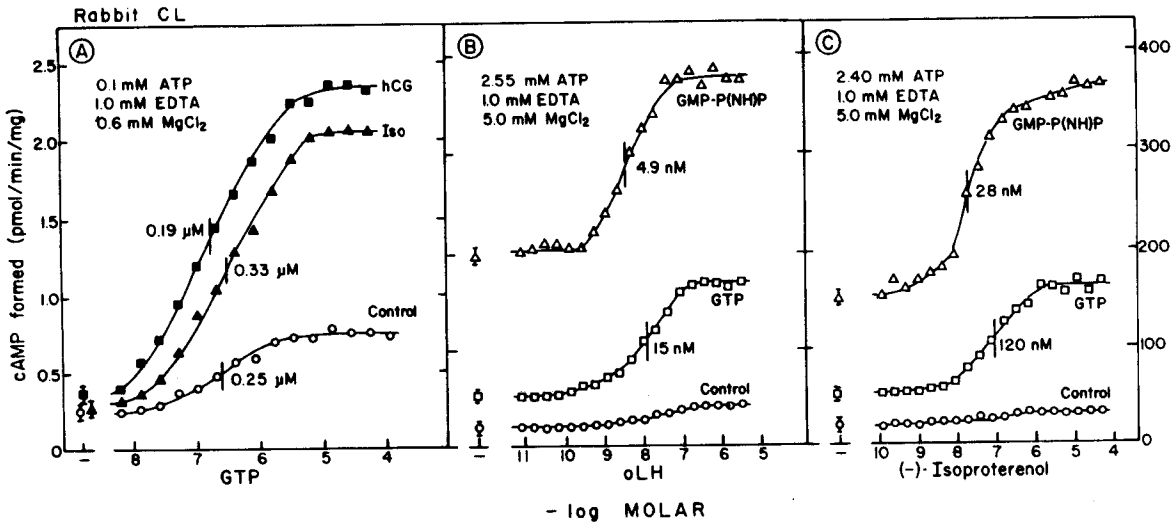

FIG. 5. A, GTP-mediated stimulation of rabbit luteal adenylyl cyclase by isoproterenol and hCG. Assays were determined at 32.5 C for 10 min in the presence of 0.1 mM ATP, 0.6 mM $MgCl_2$, and a nucleoside triphosphate-regenerating system which had been purified to remove contaminating GTP-like material. When present, the hCG concentration (■) was 10 μg/ml, and the isoproterenol level (▲) was 100 μM. The membrane protein content was 10 μg/50-μl assay. For the rest of the conditions, see *Materials and Methods*. B and C, Effects of GTP and GMP-P(NH)P on concentration-effect curves for LH (B) and isoproterenol (C). Assays were determined at 32.5 C for 10 min in the presence of the indicated concentrations of ATP, 5.0 mM $MgCl_2$, and a nucleoside triphosphate-regenerating system, which had been purified to remove contaminating GTP-like material. The membrane protein content was 5 μg/50-μl assay. The *vertical lines and values next to the curves* represent K_{act} values. When present, GTP and GMP-P(NH)P concentrations were both 10 μM.

tography (ATP), charcoal adsorption (creatine phosphate), and gel filtration (creatine phosphokinase and myokinase) as well as low concentrations of membranes, it became apparent that the luteal adenylyl cyclase is under the control of guanine nucleotides in terms of both basal activity and its responsiveness to gonadotropins and catecholamines (Fig. 5). Thus, in the absence of any guanine nucleotide addition, saturating concentrations of LH, hCG, and isoproterenol stimulated rabbit luteal adenylyl cyclase activity only marginally. The addition of saturating concentrations of GTP resulted in a 3.5- to 4.5-fold stimulation adenylyl cyclase activity due to the presence of hormones. In contrast, in the presence of 10–100 μM GMP-P(NH)P, LH and isoproterenol produced only a 2- to 3-fold stimulation of enzymatic activity over that obtained with GMP-P(NH)P alone.

The presence of GTP and GMP-P(NH)P in the assay altered the apparent affinity for LH and isoproterenol to activate adenylyl cyclase activity (Fig. 5, B and C). The concentrations of LH and isoproterenol required to half-maximally stimulate adenylyl cyclase activity (K_{act} values) were higher in the presence of 10 μM GTP than in the presence of 10 μM GMP-P(NH)P. Thus, concentration-effect curves for LH and isoproterenol in the presence of GMP-P(NH)P were to the left of those obtained with GTP.

Figure 6 depicts the effects of LH and isoproterenol on the time course of cAMP accumulation in the presence of 100 μM GMP-P(NH)P and low Mg. As seen with increasing Mg ion concentrations (Fig. 2), the addition of LH and isoproterenol to a time course of cAMP accumulation in the presence of GMP-P(NH)P resulted in a reduction in the time required to reach steady state velocity from 12 min in the absence of hormone to about 3 min in the presence of hormone. LH and isoproterenol also increased the steady state velocity of the reaction by approximately 2-fold. Thus, both hormones, and by inference both types of hormone receptors, regulate the luteal adenylyl cyclase in similar, if not identical, ways; they mimic the action of high concentrations of Mg, they stimulate the system only if guanine nucleotides are present, their concentration-effect curves are affected equally by GTP and GMP-P(NH)P, and they stimulate maximally over the same narrow range of Mg concentration, the stimulations being of similar magnitude.

Do LH and isoproterenol activate the same luteal adenylyl cyclase?

To complete the validity of the direct comparisons between gonadotropin and catecholamine activation of luteal adenylyl cyclase made above, it was necessary to demonstrate that LH and isoproterenol activate the same adenylyl cyclase system. Table 1 shows results obtained under three different asssy conditions. It was found that stimulations of rabbit luteal adenylyl cyclase by LH and isoproterenol in the presence of GTP are partially additive, suggesting the involvement of more than one adenylyl cyclase system. However, when the same experimen

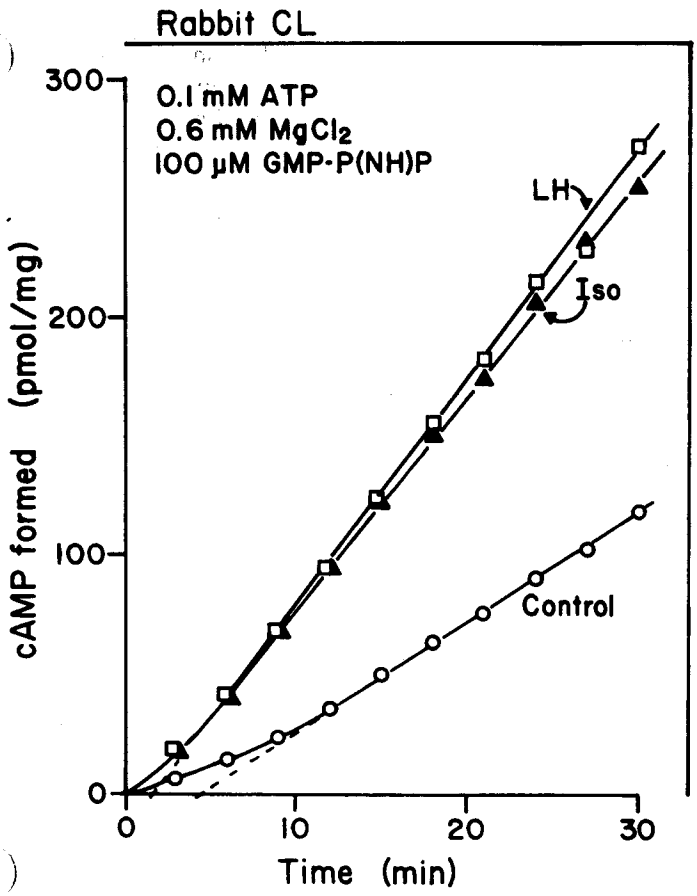

FIG. 6. Effects of LH and isoproterenol on cAMP accumulation by rabbit corpus luteum membranes in the presence of GMP-P(NH)P. Rabbit luteal membranes (200 µg/ml) were incubated in a final volume of 0.8 ml, as described in *Materials and Methods*, using 0.1 mM ATP, 0.6 mM $MgCl_2$, 1.0 mM EDTA, and 100 µM GMP-P(NH)P. At the indicated time intervals, 50-µl aliquots were withdrawn, and [^{32}P]cAMP accumulation was determined. When present, the LH level was 10 µg/ml, and the isoproterenol concentration was 100 µM. For the rest of the conditions, see *Materials and Methods*.

was performed in the presence of GMP-P(NH)P, the activities elicited by combinations of LH and isoproterenol were not additive, indicating that in these membranes, a single adenylyl cyclase system is stimulated by both gonadotropins and catecholamines. Thus, in systems such as the rabbit corpus luteum, which responds to more than one hormone and in which the combination of hormone plus GTP does not lead to full stimulation of adenylyl cyclase activity, it may be necessary to assess combined hormonal effects in the presence of a guanine nucleotide analog [*e.g.* GMP-P(NH)P] which appears to allow for full stimulation of activity upon hormone addition in order to establish whether the hormones under study activate the same adenylyl cyclase system. Assessment of additivity in the presence of GMP-P(NH)P can establish whether two hormones are activating different adenylyl cyclase systems. The luteal adenylyl cyclase is

TABLE 1. Adenylyl cyclase activities from rabbit corpus luteum membranes in response to LH and isoproterenol

Additions to assays[a]	Adenylyl cyclase activities (pmol/mg·min)[b]	
	10 µM GTP	10 µM GMP-P(NH)P
A. 0.1 mM ATP, 0.6 mM $MgCl_2$		
None	0.75 ± 0.06	2.57 ± 0.03
LH	2.80 ± 0.15 (3.73)	7.04 ± 0.22 (2.74)
Isoproterenol	2.33 ± 0.29 (3.11)	8.37 ± 1.02 (3.26)
LH plus isoproterenol	4.69 ± 0.43 (6.25)	9.20 ± 0.42 (3.58)
B. 0.1 mM ATP, 1.5 mM $MgCl_2$, 20 mM inorganic phosphate		
None	3.0 ± 0.1	8.6 ± 0.1
LH	10.1 ± 0.6 (3.37)	21.3 ± 0.5 (2.45)
Isoproterenol	9.7 ± 0.2 (3.23)	21.8 ± 0.5 (2.53)
LH plus isoproterenol	14.1 ± 0.9 (4.70)	22.3 ± 0.7 (2.59)
C. 2.67 mM ATP, 5 mM $MgCl_2$		
None	35.3 ± 2.0	158.8 ± 6.3
LH	184.3 ± 5.9 (5.22)	418.7 ± 29.0 (2.64)
Isoproterenol	149.0 ± 13.0 (4.22)	460.3 ± 10.8 (2.90)
LH plus isoproterenol	222.4 ± 13.1 (6.30)	473.2 ± 24.0 (2.98)

[a] When present, the LH level was 10 µg/ml, and the isoproterenol concentration was 100 µM. Both of these concentrations elicit a maximal response in rabbit luteal membranes.

[b] Adenylyl cyclase activities were assayed for 10 min at 32.5 C in the presence of the indicated concentrations of ATP and Mg, as described in *Materials and Methods*. The membrane protein content was 10 µg/assay. Values represent the means ± SD of triplicate determinations. Fold-stimulations (in parentheses) were calculated by dividing each experimental value by its appropriate control value.

TABLE 2. Adenylyl cyclase activities from rabbit corpus luteum membranes in response to PGE_1 and isoproterenol

Additions to assays[a]	Adenylyl cyclase activities (pmol/mg·min)[b]	
	20 µM GTP	20 µM GMP-P(NH)P
None	38.8 ± 0.2	128.6 ± 22
PGE_1	109.0 ± 5.5 (2.81)	204.7 ± 7.2 (1.59)
Isoproterenol	75.3 ± 2.0 (1.94)	195.8 ± 2.8 (1.52)
PGE_1 plus isoproterenol	137.8 ± 3.9 (3.55)	346.4 ± 4.6 (1.84)

[a] When present, the PGE_1 level was 10 µg/ml, and the isoproterenol concentration was 100 µM. Both of these concentrations elicit a maximal response in rabbit luteal membranes.

[b] Adenylyl cyclase activities were assayed for 10 min at 32.5 C in the presence of 0.1 mM ATP and 5.0 mM $MgCl_2$, as described in *Materials and Methods*. The membrane protein content was 3.8 µg/assay. Values represent the mean ± SD of triplicate determinations. Fold-stimulations (in parentheses) were calculated by dividing each experimental value by its appropriate control value.

also responsive to PGE_1 (3, 6, 9). Combinations of PGE_1 and isoproterenol in the presence of GMP-P(NH)P elicited a partially additive response (Table 2), a finding that indicates the involvement of more than one adenylyl cyclase system. As mentioned in the *Introduction*, similar results were obtained upon combining PGE_1 and LH (not shown).

Discussion

Evidence that was available when the experiments reported here were initiated (2–4) suggested strongly that either there was a specific ATP requirement for optimal LH stimulation or that in a manner analogous to the turkey erythrocyte adenylyl cyclase system, the luteal adenylyl cyclase might be different from that of most hormone-responsive systems. Further, no information was known on the characteristics of luteal catecholamine

responsiveness.

The experiments presented here show that the basic adenylyl cyclase system of rabbit corpora lutea is not of the type found in turkey erythrocytes; rather, it is of the mammalian type, such as that found in liver membranes and S49 cells. Thus, contrary to what is seen in turkey erythrocyte membranes (22, 23), luteal adenylyl cyclase exhibits a significant basal activity and is stimulated by the addition of guanine nucleotides in the absence of hormonal stimuli. As in the liver and other systems, the lags in the activation by various guanine nucleotides are nucleotide specific (24), indicating that the rate-limiting step in activation by these nucleotides is not a common step, such as would occur if the dissociation of GTP were rate limiting and, hence, responsible for the lag in the activation of the nucleotide. If this were so, then all nucleotides should stimulate with identical time lags, which they do not. As shown in Fig. 2 and 6, the luteal adenylyl cyclase system resembles other mammalian systems in that its activation by GTP analogs, such as GMP-P(NH)P, is under the control of Mg ion, and hormonal stimuli mimic the effect of Mg ion in accelerating the activation of the system by guanine nucleotides (20, 25, 26).

The experiments presented also show clearly that there appears to be no major qualitative difference between LH- and catecholamine-mediated stimulation. Both are stringently dependent on the addition of guanine nucleotides, and both exhibit a striking requirement for the presence of a very narrow range of Mg ion concentrations. In fact, such a marked dependence of hormonal stimulation relative to basal activity on Mg ion had not previously been noted in any other adenylyl cyclase system and indicates that the coupling process intervening between hormonal occupancy of receptor and adenylyl cyclase stimulation is very delicately balanced in terms of its requirements for guanine nucleotides and Mg ion. It is of interest that Lefkowitz and collaborators (27, 28) have recently proposed, on the basis of correlations between intrinsic activity and affinity of a variety of catecholamine analogs as seen in frog erythrocyte receptor-binding assays, that the formation of a given high affinity binding form of receptor is likely to be an obligatory intervening step in the signal transduction process. Bird and Maguire (29) have shown that this high affinity form of receptor is formed under the influence of Mg ion and is dependent on the presence of an intact adenylyl cyclase system. Further, guanine nucleotides decrease the abundancy of this high affinity form of the receptor by leading to formation of a low affinity form (30–32), and excess Mg promotes activation of the adenylyl cyclase complex by guanine nucleotide in the absence of hormone. Thus, the extremely narrow range in Mg concentrations that leads to optimal relative stimulations by catecholamines and LH is clearly in agreement with a dynamic situation such as that implied by these studies on the frog erythrocyte system where both Mg and guanine nucleotides are necessary for proper stimulation of adenylyl cyclase. In such a system, excess Mg ion would be inhibitory because of its action to stabilize a form of the hormone-receptor complex that is required to change under the influence of guanine nucleotide and because of the intrinsic effect of Mg enhancing basal activity.

LH and catecholamine stimulations differed in the present experiments only in as much as catecholamine stimulation was less susceptible to inhibition by excess Mg ion than LH stimulation. The reason(s) for this difference is not clear at this moment. Clearly, the earlier observation (3, 4) that LH stimulation is uniquely dependent on ATP was due to the Mg-buffering effect of ATP rather than to a specific action of ATP on the LH receptor-luteal adenylyl cyclase-coupling process.

In summary, we described basic properties of the rabbit luteal adenylyl cyclase in terms of its responsiveness to various guanine nucleotides, the modulation of this response by Mg ion, and the similar actions of occupied LH and catecholamine receptors in accelerating the activation of the luteal adenylyl cyclase system by guanine nucleotides. We conclude that both hormones activate this luteal adenylyl cyclase system through a coupling process that is qualitatively equivalent to that found in many other mammalian adenylyl cyclases in as far as its modulation by guanine nucleotides and Mg ion is concerned. The rather surprisingly narrow range of Mg ion concentrations over which high relative stimulations by hormones are obtained may relate to a combination of actions of Mg that affect both the receptor-hormone interaction and the guanine nucleotide-adenylyl cyclase interaction. Experiments are currently under way to explore the properties of the luteal catecholamine and LH receptors in terms of their regulation by Mg and guanine nucleotides.

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